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Partial Characterization of the Extracellular Polysaccharides Produced by Mutants of Rhizobium leguminosarum and Rhizobium trifolii

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Partial Characterization of the Extracellular Polysaccharides produced by Mutants of *Rhizobium leguminosarum* and *Rhizobium trifolii*

(TITLE)

BY

Lendell L. Cummins

THESIS

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ABSTRACT

Extracellular polysaccharides (EPS) are suspected to be involved in some aspect of the *Rhizobium*-legume symbiotic process. Extracellular polysaccharides produced by *R. leguminosarum* and *R. trifolii* mutants were isolated and partially characterized by gas chromatography, column chromatography, double diffusion, polyacrylamide gel electrophoresis, and immunoelectrophoresis.

The *R. leguminosarum* mutants, derived from strain 128C53, all are deficient in EPS production (exo-). Strain ANU54 is missing the symbiotic plasmid, while plasmid-containing strains ANU54 pJB5JI and ANU54 pBR1AN nodulate (nod+) peas and clover respectively, but do not fix nitrogen (fix-).

R. leguminosarum mutants produce an EPS that is similar to parental LPS in composition. Double diffusion studies indicate that all mutant LPS and EPS are antigenically identical. Both plasmid-containing mutants show an extra precipitin line in double diffusion analysis and unique antibody-binding bands when subjected to immunoelectrophoresis.

R. trifolii ANU794 produces normal amounts of EPS (exo+) and carries on normal symbiosis with clover. ANU437 (nod+ fix-exo-), is a Tn5 mutant of ANU794, but the insertion is not in the symbiotic plasmid. Strain ANU437RP4

(nod+ fix+,exo+) is strain ANU437 which has been complimented with a 4kb region from a wild-type *R. trifolii*.

ANU437 produces an EPS that has a sugar composition similar to parental LPS. The 4kb insertion contained in ANU437RP4 restores the production of normal amounts of EPS that is similar in composition to parental EPS.

Other *R. trifolii* mutants studied are affected in their ability to cause root hair curling. Strains ANU262 and ANU258 have a Tn5 insertion in the symbiotic plasmid, and cause severe root hair curling (hac++), but ANU258 is also affected in that it now has an increased host range.

Purification of LPS by Sepharose 4B column chromatography revealed that the void and included volumes of strains ANU258 and ANU262 contain KDO and heptose, while parental strain ANU843 did not. The pyruvate level is lower in the LPS from ANU258 than in the LPS from ANU262. Double diffusion studies indicate that LPS from ANU258 and ANU262 is antigenically identical to the parental LPS and to LPS from mutants that do not cause root hair curling. The EPS composition of ANU258 and ANU262 is similar to strain ANU843.

This study suggests that some small molecular weight molecules produced by plasmid-containing mutants of *R. leguminosarum* may have a role in the formation of

nodules. Studies of *R. trifolii* EPS indicate that EPS may be important in the formation of effective nodules, but not in causing extreme root hair curling. Preliminary studies of LPS from mutants affected in root hair curling suggest that some differences exist in pyruvate levels and composition of Sepharose 4B peaks.

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To my wife, Kathy, and my children, Christopher and Jonathan, I dedicate this work. They have often been without a husband or father in order for my graduate work to be completed. Their days have often been long, and their work hard, but I have always had their support. I thank them, for without their contribution, I would not have made it.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Nitrogen is perhaps the most limiting macronutrient in plant growth and development. Without sufficient available nitrogen, plants soon show typical deficiency symptoms which include chlorosis and a spindly appearance. Nitrogen is typically supplied to crop plants in the form of various commercial fertilizers such as anhydrous ammonia or urea. Organic materials such as manures or plant debris applied to the soil provide a slow release of nitrogen as a result of the decomposing action of various microorganisms present in the soil. Certain microorganisms are able to convert atmospheric dinitrogen into ammonia, which is available to higher plants. Examples of such organisms are *Clostridium pasteurianum*, *Azotobacter sp.*, and *Rhizobium sp.* *Clostridium pasteurianum* and *Azotobacter sp.* are free-living organisms that obtain their energy requirements for nitrogen fixation from carbon sources in the soil. *Rhizobium*, however, in addition to being a free-living organism, can also form a symbiotic relationship with plants in the family Leguminoseae. Only when a symbiotic relationship has formed is *Rhizobium* able to fix atmospheric nitrogen, which is done at the expense of host photosynthate (2). Because of nitrogen's importance as a macronutrient, and because of the increasing

cost of commercial fertilizer, the biological process of nitrogen fixation has become a topic of much research. *Rhizobium* is especially interesting because of its unique symbiotic relationship with the host plant. Not only does it fix nitrogen, but it accomplishes this process with energy captured via photosythesis. The advent of genetic engineering techniques has led to the hope that *Rhizobium* can be altered to enable it to carry out nitrogen fixation with increased efficiency with its normal host, or with non-legume plants.

Rhizobium is usually very specific in its ability to effectively infect a host plant. *Rhizobium trifolii*, for example will nodulate clover, but will not nodulate soybeans, which are only nodulated by *R. japonicum*. *Rhizobium leguminosarum* nodulates peas, *R. phaseolus* beans, and *R. meliloti* alfalfa. The understanding of this host specificity is of great interest, not only from a basic biological point of view, but also because understanding host recognition events is important in the development of improved strains of *Rhizobium*.

The process of nodulation of the host legume by a species of *Rhizobium* consists of several distinct steps. The first interaction between the host plant and *Rhizobium* apparently is attachment of the bacterium to a root hair (2). This attachment may be the result of an interaction preceding attachment, eg., the induction of

surface molecules on the plant by the bacterium, or induction of surface molecules on the bacterium by the plant. It has been hypothesized that the attachment of *Rhizobium* to a host's root hair is mediated by host plant lectins (carbohydrate binding proteins), and that the specificity of the *Rhizobium*-legume interaction is determined by the interaction of cell-surface polysacharides on *Rhizobium* and lectins present on the surface of the root hairs of the host plant (36).

After successful attachment, the bacteria induce curling and branching of the root hairs. Curling results in the envelopment of the bacteria by the root hair, followed by the formation of a narrow tubular structure (called the infection thread) that exends from the point of infection, through the hair cell, and into the cortical region of the root. The bacteria pass through the infection thread, are enclosed by host plasma membrane, and are released into the cortical region of the root where they undergo morphological changes. This altered form of *Rhizobium* is referred to as a bacteroid, and is the form that successfully interacts with the host plant to fix nitrogen (2). Bacteria must successfully complete each stage of the infection process in order to form effective nodules.

The specificity of *Rhizobium*-legume interactions presumably occurs as a result of the interaction of some surface or secreted molecule(s) on the bacteria with molecule(s) on the surface of the host root. Another

possibility exists in that surface or excreted molecule(s) on the bacteria may prevent recognition of the bacteria as pathogens in one or more of the infection steps, thus preventing a host response, and enabling nodule formation to occur. Bacterial molecules that may be involved in symbiotic specificity include extracellular polysaccharides (EPS), lipopolysaccharides (LPS), and capsular polysaccharides (CPS).

Rhizobium is a gram-negative bacterium with a characteristic outer membrane. In this membrane is embedded the lipid portion of the LPS, connected to the O-antigen (polysaccharide) portion by the core region (41). The lipid is attached to the core region via an acid-labile 2-keto-3-deoxyoctonic acid (KDO) linkage (10). Immediately exterior to the outer membrane lies the capsule, a region of tightly adhering polysaccharide. Excreted polysaccharides (EPS) are found in the surrounding medium. It has been suggested that one or more of these surface polysaccharides is/are involved in establishing a successful symbiosis.

Since *Rhizobium* typically produces copious quantities of EPS, it has been hypothesized to be an important determiner in establishing host specificity (10). However, the EPS compositions of *R.trifolii, R. leguminosarum, and R. phaseoli* are nearly identical, and have the same or nearly identical structures (10). The compositional and structural data does not support EPS as

the prime determiner of host specificity, but subtle differences such as the position of attachment of pyruvate and/or acetate groups may be important in determining specificity. The lectin hypothesis holds that the host lectin specifically binds *Rhizobium* via a lectin-carbohydrate interaction. Several reports have shown that EPS is bound by host lectin, perhaps constituting the initial recognition event in symbiosis (13).

Studies of the possible involvment of LPS in establishing a successful symbiosis are inconclusive. Lipopolysaccharides vary in structure between strains of *R. trifolii, R. leguminosarum*, and *R. phaseoli* (10). Lipopolysaccharide has been observed to bind host lectin (13). Mutant strains of *Rhizobium* resistant to a bacteriophage specific for LPS were found to be altered in some aspect of symbiosis (3,15,16).

Extracellular polysaccharides, CPSs, and LPSs have been shown to specifically bind host lectins (2,5,6,7,13,32,37). Fluorescein-labeled antibody and fluorescein-labeled lectin bind in a polar fashion to *Rhizobium* (9,13,35). Fluorescein-labeled clover lectin, trifoliin, polarly binds to capsular material of *R. trifolii* which, in turn has been visualized by phase contrast microscopy to bind polarly to clover root hair (13). The binding of soybean lectin to *R. japonicum* was reported to be transient in nature, with binding occurring during the mid-log phase of growth (27). Some capsule-less strains of *R. japonicum* that

previously did not bind soybean lectin (but did form nodules) were induced to bind soybean lectin when grown in the presence of roots, root exudate, or soil extracts (33). Lipopolysaccharide from a strain of *R. leguminosarum* was reported to bind specifically to pea lectin, and LPS from a strain of *R. japonicum* binds specifically to soybean lectin (23).

The lectin hypothesis is not supported by the report of nodulation in soybeans that do not contain lectin (29). Furthermore, some fast growing strains of *R. japonicum* with the ability to nodulate soybeans do not bind soybean lectin (18).

Genetically, many symbiotic functions have been attributed to plasmids, including nodulation, nitrogen fixing ability, host range, and ability to cause root hair curling (4,20,22,28). This knowledge of *Rhizobium* genetics has facilitated the study of the symbiotic process by enabling the construction of genetically well-defined mutants.

Since many of the symbiotic functions have been assigned to plasmid-borne genes, the isolation and characterization of *Rhizobium* species containing mutated plasmids is an important step in understanding the symbiotic relationship. The creation of mutated plasmids has been accomplished by a process called transposon mutagenesis (30). Transposons are short DNA sequences that are able to insert themselves into host DNA (either

chromosomal or plasmid) randomly (25). The successful insertion of a transposon into DNA is usually determined by selecting for antibiotic resistance that transposons such as Tn5 carry. Insertion of a transposon into a particular DNA sequence destroys the gene into which it has inserted. By isolating transposon mutants that have insertions in areas of interest (i.e., the nodulation region of the symbiotic plasmid), it may be possible to characterize the nature of the mutation in terms of differences in the surface molecules between the parent and mutant.

A transposon generated mutant used in this study is *R. leguminosarum* ANU54 pJB5JI. Plasmid pJB5JI is a Tn5 derivative of the bacteriocinogenic plasmid pRL1JI (4). When inserted into *R. leguminosarum* ANU 54 which does not produce extracellular polysaccharide and does not nodulate the host, pea, it confers the ability of the bacterium to form nodules on its host, pea, but not to fix nitrogen (B. G. Rolfe, personal communication). Plasmid pBR1AN, a plasmid isolated from a *R. trifolii* strain, when inserted into *R. leguminosarum* ANU54, enables the bacterium to form nodules on clover, but not to fix nitrogen. The parent strain to *R. leguminosarum* ANU54 is *R. leguminosarum* 128C53 which produces normal amounts of extracellular polysaccharide, and forms effective nodules with its host, pea.

Several mutants of *R. trifolii* strain ANU843, which causes root hair curling (hac+) and successfully

nodulates (nod+) the host, clover, have been studied. Strain ANU851 has a Tn5 insertion into the nod region of the symbiotic plasmid and is hac-nod-(B. G. Rolfe, personal communication). Strain ANU845 has been heat cured of its symbiotic plasmid and is hac-nod-(B. G. Rolfe, personal communication). ANU845 pRT150 is derived from ANU845 by insering part of the nod region of the symbiotic plasmid of ANU843 into its genome. This bacterium now causes extreme root hair curling (hac++), but is still nod- (B. G. Rolfe, personal communication). Strain ANU258 is a Tn5 mutant of the symbiotic plasmid. Its phenotype is hac++nod+, and it has an increased host range (B. G. Rolfe, personal communication). ANU262 has a Tn5 insertion in the symbiotic plasmid, and has a resulting phenotype that is hac++nod- (B. G. Rolfe, personal communication). *Rhizobium trifolii* ANU794 (hac+nod+) is the parent strain to another set of mutants. ANU437 is a Tn5 mutant of ANU794, but the insertion is not in the symbiotic plasmid. The mutation changes the phenotype to nod+, but the nodules are ineffective at fixing nitrogen (fix-) (B. G. Rolfe, personal communication). Strain ANU437RP4 (nod+fix+) is strain ANU437 which has been complimented with a 4Kb region from a wild-type *R. trifolii* strain (B. G. Rolfe, personal communication).

The purpose of this study is to compare the extracellular polysaccharides of *R. leguminosarum* 128C53 and *R. trifolii* 843 mutants described above.

Any differences noted may help assign a role to these molecules in the *Rhizobium*-legume symbiotic process.

EXPERIMENTAL

Bacterial Growth All *Rhizobium leguminosarum* strains were grown in Vincent's medium (38) consisting of: Mangnesium sulfate, 150mg/l; calcium chloride, 50 mg/l; potassium phosphate (dibasic), 600 mg/l; ferric chloride, 1.8 mg/l; sodium chloride, 60 mg/l; biotin, 200 mg/l; thiamin HCl, 100 mg/l; calcium pantothenate 100 mg/l; mannitol, 10 g/l; glutamic acid, 1.1 g/l.The pH was adjusted to 6.8 with NaOH prior to autoclaving. Starter cultures (40ml) were used to inoculate four 500 ml cultures with equal amounts of bacteria (equal O.D. units measured at 620 nm) for each *R. leguminosarum* strain grown. The 500ml cultures were grown to early stationary phase on a shaker at room temperature (25 degrees Centigrade). All strains were checked for contamination and reversion at each transfer and before harvesting by gram staining, absence of growth on nutrient agar, and growth on media containing proper antibiotics (Table I).

All *R. trifolii* strains were grown in New Modified Bergersen's Medium consisting of: Potassium phosphate (dibasic), 250mg/l; magnesium sulfate, 100mg/l; glutamic acid, 1.1g/l; mannitol, 10g/l; calcium pantothenate, 4mg/l. TRACE ELEMENTS : Calcium chloride, 6.62mg/l; boric acid, 0.145 mg/l; cobalt chloride, 0.059 mg/l ferrous sulfate 0.125 mg/l; copper sulfate, 0.005 mg/l;

manganese chloride, 0.0043 mg/l; zinc sulfate, 0.108 mg/l; sodium molybdate (dihydrate), 0.125 mg/l; nitriloacetate, 0.007 mg/l. VITAMINS : Riboflavin, 0.02 mg/l; p-aminobenzoic acid, 0.02 mg/l; nicotinic acid, 0.02 mg/l; biotin, 0.02 mg/l; thiamin-HCl, 0.02 mg/l, pyridoxine-HCl, 0.02 mg/l; inositol, 0.02 mg/l.

Trace elements were prepared as a 1000x stock solution and the pH was adjusted to 5.0 before adding nitriloacetate to prevent precipitation of the trace elements.

Vitamins were prepared as a 1000x stock solution in 0.05M Sodium phosphate (dibasic) buffer at a pH = 7.0.

All *R. trifolii* strains were grown as 8 - 12 liter aerated cultures, and harvested at early stationary phase. Each batch was checked for contamination as previously described.

ISOLATION OF EXTRACELLULAR POLYSACCHARIDES (EPS)

Bacteria were centrifuged out of the media at 15,000 xg for 15 minutes. The supernatant was filtered through a 0.8 micron filter, followed by a 0.45 micron filter. The filtered superntant was then concentrated by rotary evaporation at 40 degrees Celsius under reduced pressure to a volume of approximately 100ml. Three volumes of ethanol were added, the precipitate was collected be centrifugation, dissolved in deionized water, dialized against deionized water for at least four changes of water at six hour intervals, and then freeze-dried.

ISOLATION OF LIPOLYSACCHARIDES (LPS) Bacterial LPS was isolated by the hot phenol/water method (40). A 100ml suspension of bacteria in deionized water at 65 C was mixed with phenol at 65 C for 15 minutes, cooled for 15 minutes in an ice bath, then centrifuged at 15,000 xg for 20 minutes. The top water layer containing the LPS was removed, and the procedure was repeated using a fresh supply of water. The collected water layer was dialized against deionized water changing the water at least four times at 6 hr. intervals, treated with 0.1ml of DNase and 10ml of RNase per 100ml solution, allowed to stand for 24 hrs. at room temperature, then dialized against deionized water for at least four changes of water at 6 hr. intervals, and freeze-dried.

PURIFICATION OF POLYSACCHARIDES

Purification of EPS and LPS was accomplished by column chromatography using a Sepharose 4B column equilibrated with EDTA/triethylamine (10mM/30mM). Peaks were detected by the anthrone assay for hexose (14). Any peaks detected were collected, dialized against deionized water, and freeze-dried.

PREPARATION OF ANTISERUM

Rhizobium leguminosarum strains ANU 54, pJB5JI, and pBR1AN were grown to stationary phase in Vincent's medium and harvested by centrifugation. The bacterial pellets were suspended in a 0.05% formalin-PBS solution (1.4ml of 37% formalin, 0.268 g Na2HPO4, 0.85 g NaCl per 100ml, pH

adjusted to 7.0 with HCl). The bacterial suspensions were diluted with formalin-PBS solution to an absorbance of 0.58-0.60 at 620 nm. The bacterial preparations were checked for sterility by streaking on solid media. Intravenous injections into New Zealand white rabbits of 0.5 ml were made according to the following schedule: All strains were injected on day 1, 5, 9, 14, and 18. On day 25, antiserum to ANU54 and ANU 54 pJB5JI was collected, and another injection of ANU54 pBR1AN was made. Antiserum to ANU54 pBR1AN was collected on day 34. For all strains, the blood was allowed to stand at room temperature for 2 hours, then placed in a cold room overnight. The blood was centrifuged on a tabletop centrifuge, and the antiserum (supernatant) collected and frozen in 0.5 or 1.0ml aliquots.

IMMUNODIFFUSION PROCEDURE

Microscope slides were covered with a solution containing 1.5% Noble agar and 0.02% sodium azide in deionized water to a depth of 1mm. Plexiglass squares (25 x 25mm) containing five wells were placed directly upon the agar-covered slides. The center well was always used for the antiserum, with antigens placed in the others. Plates were incubated in a moist environment for 4-7 days, washed 1 hr. in PBS (0.01M Na_2HPO_4, 0.85% NaCl, 0.02% NaN_3, pH 7.0) for at least 3 changes, stained with a solution of 500/500/100 (deionized water/methanol/acetic acid) and 0.1% Commassie blue, and then destained in 800/100/100 (deionized water/methanol/ acetic acid).

ANTIGEN_PREPARATION

EPS and LPS were purified as described and applied in a 1 mg/ml solution. Bacterial solutions were prepared by grinding frozen cells, followed by sonication for 15 minutes. This procedure was repeated three times.

KDO_(2-keto-3-deoxyoctonic_acid)_ASSAY (39)

Standards of 0-75 micrograms KDO and samples were prepared in a total volume of 200 ul. Twenty microliters of 0.4N sulfuric acid was added. Then the tubes were heated for 30 minutes in a hot water bath. Two hundred fifty microliters of 0.04N periodic acid was added, the samples allowed to sit at room temperature for at least 40 minutes, then 500ul of 2% Sodium arsenite in 0.5N HCl was added, and the tubes allowed to stand for 5 minutes. Two ml of 0.3% thiobarbituric acid was added, the tubes heated for 20 minutes in a hot water bath, and the absorbance was read immediately at 548nm. Cloudy samples were centrifuged for 1 minute in a tabletop centrifuge prior to measuring absorbance.

ACETYL_ASSAY (19)

Standards (B-D(+) glucose pentacetate) and samples were diluted with methanol to 100ul, then to 400ul with deionized water. Eight-tenths ml of a solution containing equal parts of 2M hydroxylaminehydrochloride and 3.5N NaOH was added to the standards and samples. The tubes were allowed to stand for 1 minute. Four-tenths ml of a solution of 1 part concentrated HCl and 2 parts water was added, followed by

0.4ml of 0.37M Ferric chloride in 0.1N HCl. The absorbance was read at 540nm.

PYRUVIC_ACID_ASSAY (24)

Standards (pyruvic acid) and samples were prepared in screw-cap test tubes to a total volume of 200ul. Three hundred microliters of 2.0N HCl was added and the tubes sealed and heated at 100 C for 3 hours. One hundred microliters of 5.0mM 2,4-dinitrophenylhydrazine in 2.0N HCl was added and the tubes allowed to stand at room temperature for 30 minutes. Six hundred microliters of toluene was added, the tubes mixed, and the bottom water layer removed. Six hundred microliters of 10% Sodium carbonate was added to the toluene, the tubes mixed, and the toluene layer discarded. Four hundred microliters of deionized water and 1.0ml of 2.2N NaOH were added to the remaining solution. Absorbances were read at 416nm after centrifugation for at least 1 minute in a tabletop centrifuge.

URONIC_ACID_ASSAY (8)

Standards (glucuronic acid) and 2 sets of samples were prepared to a total volume of 200ul. 1.2ml of cold 0.125M sodium tetraborate in concentrated sulfuric acid was added to all tubes. The tubes were heated for 5 minutes in a boiling water bath, then cooled for 1-2 minutes in cold water. To the standards and to one set of samples, 20ul of 0.15% m-hydroxybiphenyl in 0.5% NaOH was added. 20ul of 0.5% NaOH was added to the other set of samples. The tubes were allowed to stand for 5 minutes at room temperature.

Absorbance was read at 520nm. Any reading from the NaOH samples was subtracted from that of the m-hydroxybiphenyl readings.

ACTETYLATION PROCEDURE (1)

A standard solution containing 1mg/ml of rhamnose, fucose, xylose, mannose, galactose, glucose, and heptose was prepared. One hundred microliters of the standard sugar solution was added to a screw-cap test tube. Samples of not more than 250ug of hexose (determined by anthrone assay) were placed in screw-cap test tubes. Twenty microliters of a 1mg/ml inositol solution was added to all tubes as an internal standard. Samples and standards were dried using filtered air or by freeze-drying. Five hundred microliters of 2M trifluoroacetic acid was then added, the tubes sealed with teflon-lined screw caps and heated at 121 C for 2 hours. The samples and standards were then dried with filtered air at 40 C, 250ul of a 10mg/ml solution of sodium borohydride in 1M ammonium hydroxide was added to all tubes, and the tubes allowed to stand at room temperature for 1 hour. One hundred fifty microliters of glacial acetic acid was added to all tubes in 50ul aliquots. Five hundred microliters of a 9:1 methanol:acetic acid solution was added to all tubes and the samples dried with filtered air. This step was repeated 4 times. Five hundred microliters of methanol was added to each tube and the samples dried with filtered air. This step was repeated 4 times. Fifty microliters of pyridine and 50ul of acetic anhydride was

added to all tubes, the tubes heated at 121 C for 30 minutes, then cooled on ice. Samples were extracted with 500ul of water and 500ul of chloroform, the chloroform layer was saved and dried with filtered air. Samples were analyzed by gas chromatography with a Hewlett Packard gas chromatograph and a Supelco SP2330 capillary column. Helium was used as the carrier gas, detection was accomplished with a flame-ionization detector (hydrogen-compressed air flame),and the data collected and analyzed with an Apple IIe microcomputer and Chromatochart (I.M.I., Inc., State College, Pa.) software package.

POLYACRYLAMIDE GEL ELECTROPHORESIS (12)

Discontinuous polyacrylamide gel electrophoresis (PAGE) was used to analyze samples of EPS and LPS. Separation was done using either a linear gradient gel (10-20% or 10-17%), or on a 15% gel, using a 5% stacking gel. Gels were run using a Buchler 3-1500 power supply at a constant 20mA.

STOCK SOLUTIONS

Stock solutions were prepared as follows:

Running gel stock buffer

Tris base: 22.71 grams.

Deionized water 75ml.

pH adjusted to 8.8 with HCl

Total volume adjusted to 100 ml with deionized water.

Stacking gel stock buffer

Tris base 7.69 grams.

Deionized water: 75ml.

pH adjusted to 6.8 with HCl.

Total volume adjusted to 100 ml with deionized water.

Stock acrylamide solution

Acrylamide: 25 grams.

Bis acrylamide: 0.625 grams.

Total volume adjusted to 100ml with deionized water.

Sodium docecylsulfate (SDS) solution (10%)

SDS: 2.5 grams.

Deionized water: 25ml.

Sample buffer

Stacking gel stock buffer: 5ml.

Sucrose: 2.5 grams.

SDS: 0.5 grams.

Mercaptoethanol: 1.25ml.

Brom-phenol blue: 0.25 grams.

Total volume adjusted to 25ml with deionized water.

Running buffer

Tris base: 3.0 grams/liter.

Glycine: 14.4 grams/liter.

SDS: 1.0 gram/liter.

Preparation of running gel

1. 15% acrylamide gel:

10ml deionized water

6ml stock acrylamide

4ml running gel buffer

0.2ml 10% SDS

Just before pouring the gel, 40ul of 10% ammonium

persulfate and 10ul of TEMED were added.

2. Gradient gel preparations:

10% acrylamide gel:

6ml deionized water

2ml stock acrylamide

2ml running gel buffer

0.1ml 10% SDS

17% acrylamide gel:

4.6ml deionized water

3.4ml stock acrylamide

2ml running gel buffer

0.1ml 10% SDS

20% acrylamide gel:

4ml deionized water

4ml stock acrylamide

2ml running gel buffer

0.1ml 10% SDS

Just prior to pouring, 20ul of 10% ammonium persulfate and 5ul of TEMED were added to the 10% acrylamide solution and either the 17% or the 20% acrylamide solution.

Stacking gel preparation

2ml of stacking gel stock buffer

1ml of stock acrylamide

0.1ml of 10% SDS

6.9ml deionized water

Just prior to pouring, 20ul of 10% ammonium persulfate and 5ul of TEMED was added.

SAMPLE_PREPARATION

Samples were prepared by freeze-drying 25-40ul of 1mg/ml solutions, then treating with 30ul of sample buffer, and heating at 100 C for 5 minutes in sealed tubes.

Staining_procedure

1. After running, gels were soaked overnight in 25%/10%/65 isopropanol/acetic acid/water.

2. Oxidation was accomplished using a solution of 1.17 grams sodium meta periodate, 4ml of 25%/7%/68% (isopropanol/acetic acid/water), and 150ml deionized water for 5 minutes.

3. The gels were washed in deionized water 3 times for 1 hour each wash.

4. The gels were stained for 10 minutes with a solution of 28ml of 0.1N NaOH, 1ml concentrated ammonium hydoroxide, 5ml of 20% silver nitrate, 115ml deionized water. Concentrated ammonium hydroxide was added dropwise until no ppt. remained.

5. The gels were washed in deionized water 4 times for 30 minutes each wash.

6. Gels were developed in a solution of 0.05 grams citric acid, 0.5ml 37% formaldehyde in 200ml deionized water, then washed overnight in deionized water.

ELECTROBLOT_PROCEDURE

Polysaccharide fractions resolved by polyacrylamide gel electrophoresis were transferred to nitrocellulose paper by electrophoresis using a Transphor Model TE50 transfer unit

and a buffer of 25mM Tris base, 192mM glycine, and 20% (vol/vol) methanol (42). Transfer was carried out for 1.5 hours at 75 volts (0.5-0.65 amperes). After transfer, the nitrocellulose paper was treated with a solution of Tris-buffered saline (TBS) (50mM Tris, 200mM NaCl, pH 7.4) containing 3% bovine serum albumin (BSA) for 30 minutes. Antiserum raised against *R.leguminosarum* ANU54 pJB5JI was added to this mixture to give a final antiserum dilution of 1:100. After 3 hours of incubation in antiserum, the nitrocellulose paper was washed at least 3 times with TBS for 15 minutes each wash. After washing, the paper was treated with a preparation of peroxidase-conjugated goat anti-rabbit antiserum (1:1000 dilution) in TBS containing 3% BSA followed by a final washing in TBS (at least 3 times). Antigenic components were visualized by developing in a solution of 5ml of 4-chloro-1-napthol (3mg/ml in methanol), 25ml TBS, and hydrogen peroxide (0.01% vol/vol). After developing, the paper was rinsed in deionized water and allowed to dry.

RESULTS

Yields of Extracellular Polysaccharide . The yields of EPS from the parent and mutant strains of *R. leguminosarum* and *R. trifolii* are given in Table II. The results indicate that the mutant strains of *R. leguminosarum* are severely deficient in EPS production. The presence of the symbiotic plasmid in ANU 54 pJB5JI and in ANU 54 pBR1AN does not restore the production of normal amounts of EPS, although it does enable the formation of nodules. The results for the *R. trifolii* mutants indicate that all but strain 437 produce normal amounts of EPS. The corrected strain, *R. trifolii* ANU 437RP4, which has been complimented with a 4 Kb region from a wild-type *R. trifolii* strain, produces normal amounts of parental EPS.

Sepharose 4B Column Chromatography of the Extracellular Polysaccharides . Column chromatography results, shown in Figure 1 indicate that the EPS from the *R. leguminosarum* ANU54 pJB5JI elutes as two peaks, one peak having an elution volume characteristic of LPS, the other eluting at the included volume. In an earlier study of *R. leguminosarum* ANU54, it was shown that the EPS consisted of LPS and a small molecular weight glucan (11). The results for *R. leguminosarum* ANU 54 and ANU54 pBR1AN show similar elution patterns. The parental EPS

elutes as two peaks, one at the void volume, and one at the included volume.

The results for *R. trifolii* ANU437, shown in Figure 2, show an elution pattern of three peaks, one at the void volume, one with an elution volume similar to that of LPS, and one at the included volume. The elution pattern always gives three peaks, but the amount of material in each peak can vary. The parental EPS elutes as two peaks, one at the void volume, and one at the included volume. The corrected mutant EPS elutes as one peak at the void volume.

Composition of Sepharose 4B Column Peaks . The sugar composition of the polysaccharides present in the Sepharose 4B column peaks was determined by gas chromatography after hydrolysis of the polysaccharides and conversion of the monosaccharides into their alditol acetate derivatives. The KDO, acetyl, pyruvic acid, and uronic acid present was determined as previously described. The results are shown in Table III. The composition of the *R. leguminosarum* mutant peaks corresponding to LPS are very similar to the composition of LPS, except for slightly increased levels of glucose, which may be due to incomplete separation of these peaks from the small molecular weight glucan.

Compositional analysis, shown in Table III, of the *R. trifolii* ANU 794 and *R. trifolii* 437RP4 peaks which elute at the void volume indicates that they are very similar. The compositional analysis of the void volume peak (pk 1) of *R. trifolii* ANU437, however, shows that the

same sugars are present, but at lower levels when compared to the parent and corrected mutant void volume peaks. This apparent difference may be due to the fact that we have acccounted for only a portion of the mass. When the relative amount of each sugar is considered, the amount of each sugar in the void volume peak of each strain is similar (data not shown). These results agree with the relative composition of the acidic EPS shown in Table IV. The presence of an additional component, KDO, which is commonly associated with LPS, in the void volume material of ANU437 (pk 1) is a significant difference. The ANU437 polysaccharide with an elution volume similar to LPS has a composition similar to LPS. The peak eluting at the included volume has a relative composition similar to the same parental peak, but with an increased level of glucose, uronic acid and a significant level of KDO.

The EPS of *R. trifolii* ANU794, ANU437, and ANU437RP4 was separated into acidic EPS and neutral EPS by treatment with cetyltrimethyl ammonium bromide (CTAB). The composition of each fraction was determined as previously described. The acidic EPS results, shown in Table IV, indicate that the acidic EPS composition is similar to the composition of the void volume polysaccharide, with the exception that the ANU437 EPS pk 1 material also contains KDO. The CTAB EPSs contained mannitol at low levels, which may be due to contamination from media. The acidic material contains low levels of mannitol, galactose and glucose in

similar amounts as found in the void volume peak. The glucose:galactose ratio was between 4 & 5 to 1 in all void volume peaks, and all CTAB material. Table 5 indicates that 84% of the ANU794 EPS and 88% of the ANU437RP4 EPS is acidic EPS, while only 35% of the ANU437 EPS is acidic. By considering the total amount of EPS produced, calculations show that ANU 437 only produces 0.3% by weight the amount of acidic EPS produced by ANU794 and ANU437RP4.

Double Diffusion Assays . Sepharose 4B elution volumes and compositional analysis presented for *R. leguminosarum* ANU54, ANU54 pJB5JI, and ANU54 pBR1AN suggest that the major component of the EPS of these strains is actually LPS. To further investigate this possibility, the peaks with elution volumes similar to LPS and purified LPS were subjected to double diffusion analysis against antiserum to all three strains. Figure 3 shows that the LPSs from all three mutant strains form precipitin bands against antiserum to ANU54 pJB5JI, and that these bands are antigenically identical. A slight precipitin band is formed against 128C53 LPS which appears to have diffused at a slower rate than the mutant LPSs, but probably is also antigenically identical to the mutant LPS. The LPS from 128C53 has been shown to be antigenically identical to ANU54 EPS (Sanders,Nature). Figure 4 shows that the Sepharose EPS peak 1 is antigenically identical to the purified LPS for strain ANU pJB5JI. Similar results are given in Figures 5 and 6 for strains ANU54 and ANU54 pBR1AN respectively. A

second, weak precipitin band is seen near the center well for the EPS (peak 1) from strains ANU54 pJB5JI and ANU54 pBR1AN, but not from strain ANU54. Thus EPS from the strains carrying the symbiotic plasmid may contain an additional antigenic component not found in the EPS from strain ANU54.

Polyacrylamide Gel Electrophoresis and Immunoblot Procedures *Rhizobium leguminosarum* mutant LPS and EPS peak 1 from each strain were subjected to polyacrylamide gel electrophoresis. The results, shown in Figure 10, indicate that the LPS and EPS peak 1 from each mutant strain exihibit very similar gel separation patterns. After transfer of the separated polysaccharides to nitrocellulose paper, the antigenic characteristics of the bands were compared using antiserum to ANU54 pJB5JI. The results, shown in Figure 11, show that all strains exhibit similar binding of the antiserum. Close inspection reveals that strains ANU54 pJB5JI and ANU54 pBR1AN have additional lower molecular weight bands that bind the antiserum, but the major lower molecular weight band does not bind antiserum.

Gel electrophoresis of the Sepharose 4B peaks of *R. trifolii* ANU794, ANU437, and ANU437RP4 indicates that ANU437 (pk 2) has a polyacrylamide separation pattern similar to the banding patterns seen for LPS (data not shown).

Analysis of EPS and LPS of hac++ strains . Compositional analysis of the extracellular polysaccharides

of *R. trifolii* ANU843, ANU258, and ANU262, shown in Table VI, indicates that both mutant strains (ANU258 and ANU262) have a sugar composition similar to the parent (ANU843). Following the phenol/water extraction of the bacteria, the water layer (W.L.) was separated into three component peaks by Sephrose 4B column chromatography, as shown in Figure 12 for strain ANU 262 (hexose assay), and Figure 13 for ANU262 (KDO assay). The first peak elutes as a broad peak just after the void volume in both the hexose and KDO assays. The second peak has an elution volume similar to that of LPS, with both the hexose and KDO peaks coinciding, while the third peak elutes near the included volume in the hexose assay. The LPS (W.L. pk 2) of the two mutants is similar to the parental LPS in relative sugar percent composition, however, the pyruvate level is reduced in strain ANU258 LPS. The relative percent composition of ANU258 and ANU262 W.L. pk 1 is very similar. A comparison of the W.L. pk 3 compositions of ANU258 and ANU262 indicates a similar relative percent composition, except that strain ANU262 has traces of mannose, galactose, 2-O-methyl-6-deoxyhexose and 3-N-methyl-3-amino-3,6-dideoxyhexose.

Figure 7 shows the results of the double diffusion procedure using *R. trifolii* ANU843 and ANU845 pRT150 bacteria and antiserum to ANU845 pRT150. Strain 843 exihibits two precipitin bands, while strain ANU845 pRT150 only shows a hint of one inner precipitin band. Figures 8

and 9 indicate that the outer precipitin band of strain ANU843 is antigenically identical to the LPS of strains ANU843, ANU845 pRT150, ANU851, and ANU845.

DISCUSSION

EPS DEFICIENT STRAINS

Rhizobium leguminosarum results.

The study of surface polysaccharides produced by *R. leguminosarum* 128C53 and its mutants, ANU54 (nod-,fix-), ANU54 pJB5JI (nod+,fix-), ANU54 pBR1AN (nod+,fix- on clover), revealed that all mutants excrete greatly reduced amounts of polysaccharide compared to the parent (Table II). The elution profile of the extracellular material (Figure 1) isolated from the mutants shows a major peak with an elution volume similar to that of LPS. Compositional analysis of the major peaks revealed that these polysaccharides contain the same sugars in the same amounts as does the lipopolysaccharide (Table III). These results differ slightly from those reported by Hustmyer (21), who reported that the relative mole percent glucose in the EPS pk1 of ANU54 pBR1AN was three times the amount found in the EPS pk1 of ANU54 and ANU54 pJB5JI. Hustmyer also reported the absence of pyruvate in ANU54 EPS pk1, whereas this study found pyruvate present. Double diffusion analysis (Figures 3 - 6) indicates that the precipitin bands formed by mutant LPS and EPS are antigenically identical. Polyacrylamide gel electrophoresis results (Figure 10) show that the EPS pk 1 of each mutant has a separation pattern that is similar to that of LPS. Hustmyer (21) reported a difference in the electrophoretic banding pattern of ANU54 pBR1AN EPS pk1 when

compared to ANU54 and ANU54 pJB5JI EPS pk1. Immunoblot results (Figure 11) show that all strains have similar antibody binding patterns, but ANU54 pJB5JI and ANU54 pBR1AN show additional small molecular weight bands that bind antibody.

These results indicate that the excreted polysaccharide is probably LPS. The differences in immunoblot binding patterns suggest that excreted material from ANU pJB5JI and ANU pBR1AN may have additional molecules. Perhaps these molecules play a role in the formation of nodules. If this were the case, then one would expect to find similar binding of antibody to parental LPS. However, no such bands are seen. Lipopolysaccharide has been reported to prevent the hypersensitive response of a plant to a pathogen (26). Such "prevention of recognition as a pathogen" would be a possible role for these molecules since the mutants that show the additional bands are able to infect the host plant and form nodules while the mutant lacking a symbiotic plasmid is unable to form nodules.

The results of this study do not support a role for EPS in attachment of the bacterium to the host root hair, in root hair curling, in formation of an infection thread, or in formation of nodules. Some smaller molecular weight LPS-like molecules, on the other hand, may be involved in some fashion in the formation of nodules.

R. trifolii results.

Results indicate that the 4kb insertion into ANU437RP4

restores its ability to produce normal parental EPS. Significant differences between the mutant, ANU437, and the parent, ANU794 include: 1. The lack of production of typical parental EPS (or the production of very small quantities of parental-type EPS that has an additional KDO component). 2. The presence of KDO and elevated levels of glucose and uronic acid in the included volume peak of ANU437. The fact that strain ANU437 can produce nodules, but does not produce normal parental EPS suggests that EPS is not required for the infection process. The production of EPS, while apparently not required for nodule formation, may have a subsequent role in establishing nodules that are able to fix nitrogen. Perhaps the presence of EPS masks any interaction between the bacteria and the host, thus preventing a post-infection pathogenic response by the host.

HAC++ STRAINS

The second set of *R. trifolii* mutants cause extreme root hair curling (hac++). Significant differences between the mutants and parent are the elution of a high molecular weight peak (peak 1) that contains KDO and heptose, and the presence of KDO and traces of heptose in peak 3. The differences in composition seen in strains ANU258 and ANU262 may be linked to the inablity of ANU262 to form nodules, to the ability of strain ANU258 to infect other hosts, or to the ability of both strains to cause extreme root hair curling. The presence of KDO and heptose in peak 1 and 3 suggests that these peaks may be additional

forms of LPS, since KDO and heptose is normally associated with LPS. Peaks 1 and 3 do not, however, contain other sugars normally found in LPS such as fucose, 3-N-methyl-3-amino-3,6-dideoxyhexose, and 2-O-methyl-6-deoxyhexose. Double diffusion studies indicate that the LPS of strains ANU 845 (hac-nod-), ANU851 (hac-nod-), and ANU845 pRT150 (hac++,nod-) is antigenically identical to the parent strain, ANU843 (hac+,nod+), Figures (8-9). Figure (7) suggests that strain ANU845 pRT150 produces a substance that masks the LPS, preventing its interaction with antiserum, since purified ANU845 pRT150 LPS produces a sharply defined precipitin line (Figure 8). This suggests that LPS, as isolated in its purified form, is not responsible for causing root hair curling, or in the formation of nodules, since LPS from hac-nod- strains and hac+nod+ strains is antigenically identical. The substance, possibly EPS, that masks the interaction between antiserum and LPS could have a role in causing the extreme root hair curling observed in the interaction between strain ANU845 pRT150 and the host. Further studies including polyacrylamide gel electrophoresis, and structural determination of the EPS and LPS may help assign a symbiotic role to these molecules.

SUMMARY

The results of this study offer several suggestions of which molecules may play key roles in the *Rhizobium - legume* symbiosis. The study of *R. leguminosarum* mutants that are severly deficient in EPS production revealed that the EPS produced was very similar to LPS. Mutants containing the symbiotic plasmid (both nod+fix-) showed extra precipitin lines in double diffusion analysis and unique antibody-binding bands when subjected to immunoelectrophoresis. These results suggest that EPS is not important in nodule formation, but that some small molecular weight molecules present in the EPS of the plasmid-containing mutants may have a role in nodule formation.

Results from the study of *R. trifolii* ANU794, ANU437, and ANU437RP4 suggest that EPS may be important in the establishment of nitrogen-fixing nodules, since strains ANU794 and ANU437RP4 both produce normal EPS and both are (nod+fix+), while strain ANU437 does not produce typical parental EPS and does not fix nitrogen, although it is able to form nodules.

Studies of the hac++ mutants, *R. trifolii* ANU258 and ANU262, suggest that differences exist in water layer peaks 1 and 3. The presence of KDO and heptose suggests that these peaks represent an altered form of LPS. These molecules may be involved in root hair curling, or the determination of host range. The LPS of strains ANU258 and

ANU262 shows a similar composition, except for a lower pyruvate level in strain ANU258. This lower level of pyruvate may also be a significant difference, possibly responsible for the increased host range in strain ANU258. The LPS from hac++ strains is antigenically identical to the LPS from hac- strains. This suggests that LPS may not have a role in causing root hair curling. Strain ANU845 pRT150 (hac++) produces a substance that masks the interaction between LPS and antiserum. This substance, possibly EPS, may be involved in causing extreme root hair curling. Further studies of the polysaccharides produced by strains ANU258 and ANU262, including polyacrylamide gel electrophoresis, immunoelectrophoresis, and structural studies may help in establishing what role, if any, these molecules play in symbiosis.

Table I

GROWTH CHARACTERISTICS OF *R. leguminosarum* STRAINS

	Growth Medium			
Strain	VIN	NA	rif,str*	rif,str,kan*
128C53	+	-	+	-
ANU54	+	-	+	-
ANU54 pJB5JI	+	-	+	+
ANU54 pBR1AN	+	-	+	+

VIN - Vincent's medium NA - Nutrient Agar
rif - rifampicin str - streptomycin
kan - kanamycin
(*) - all antibiotics were added to Vincent's medium at a concentration of 100 micrograms per ml.

Table II

YIELDS OF EXTRACELLULAR POLYSACCHARIDES

Strain	yield (mg/l)	% of parent
R. leguminosarum		
128C53	467	100
ANU54	9	1.8
ANU54 pJB5JI	11	2.4
ANU54 pBR1AN	16	3.4
R. trifolii		
ANU843	530	100
ANU258	356	67
ANU262	526	99
ANU794	688	100
ANU437	17	2.4
ANU437RP4	865	126

Table III

WEIGHT PERCENT EXTRACELLULAR POLYSACCHARIDE COMPOSITION

Strain		RHA	FUC	MAN	GAL	GLC	HEP	UA	PY	AC	KDO	2OM	3NM
R. leguminosarum													
128C53*	EPS	-	-	2	13	53	-	21	NA	NA	-	-	-
ANU54**	LPS	6	6	5	1	1	-	4	1.6	2.0	1.0	-	-
ANU54	pk1	6	9	7	2	3	-	7	.7	.7	0.9	-	-
ANU54	pk2	-	-	.6	1	7	-	8	.4	.4	-	-	-
ANU54 pJB5JI	pk1	6	9	7	2	2	-	3	.9	.8	2	-	-
ANU54 PJB5JI	pk2	-	-	TR	TR	5	-	NA	.9	1.7	-	-	-
ANU54 PBR1AN	pk1	4	7	6	3	4	-	NA	.7	1.2	2	-	-
ANU54 pBR1AN	pk2	-	-	-	2	4	-	8	NA	0.9	-	-	-
R. trifolii													
ANU794	pk1	-	-	-	9	40	-	26	11	3.4	-	-	-
ANU794	pk2	-	-	-	4	20	-	23	3	1.8	-	-	-
ANU794***	LPS	-	1	2	1	1	3	16	9	0.7	1.2	1.3	1.5
ANU437	pk1	-	-	-	2	10	-	6	3	0.6	0.8	-	-
ANU437	pk2	1	2	2	4	8	3	10	2	0.5	1.4	1.4	0.8
ANU437	pk3	-	-	-	8	56	-	15	4	2.2	0.4	-	-
ANU437RP4		-	-	-	9	38	-	15	8	1.1	-	-	-

* Data from Carlson and Lee (11)
** Data from Hustmyer (21)
*** Data from Dr. Russell Carlson (personal communication)

RHA - Rhamnose FUC - Fucose MAN - Mannose
GAL - Galactose GLC - Glucose HEP - Heptose
UA - Uronic acid PY - Pyruvate AC - Acetate
KDO - 2-keto-3-deoxyoctonic acid NA - No data
2OM - 2-O-methyl-6-deoxyhexose TR - Trace
3NM - 3-N-methyl-3-amino-3,6-dideoxyhexose

Table IV

RELATIVE PERCENT COMPOSITION OF *R. trifolii* ACIDIC EPS

Strain		2OM	RHA	FUC	MAN	3NM	GAL	GLC	HEP	UA	AC	PY	KDO
R.trifolii													
ANU794	AEPS	-	-	-	2	-	9	43	-	30	2.6	14	-
ANU437	AEPS	-	-	-	2	-	6	37	-	34	3.8	14	1.1
ANU437RP4	AEPS	-	-	-	2	-	7	43	-	29	2.9	16	-

Data from Dr. Russell Carlson (personal communication)
AEPS - acidic extracellular polysaccharide
All other abbreviations are as given in Table 3

Table V

CTAB EXTRACELLULAR POLYSACCHARIDE YIELDS

Strain		Supernatant (NEPS)	Precipitate (AEPS)
R. trifolii			
	ANU794	16%	84%
	ANU437	65%	35%
	ANU437RP4	12%	88%

NEPS - Neutral extracellular polysaccharide
AEPS - Acidic extracellular polysaccharide
CTAB - Cetyltriaethylammoniumbromide
Data from Dr. Russell Carlson (personal communication)

Table VI

WEIGHT PERCENT
EXTRACELLULAR AND LIPO- POLYSACCHARIDE COMPOSITION OF
R. trifolii hac++ Strains

Strain	2OM	RHA	FUC	MAN	3NM	GAL	GLC	HEP	UA	PY	AC	KDO
ANU843 EPS*	-	-	-	-	-	9	49	-	14	11	7	-
ANU843 LPS**	6	-	2	3	5	2	1	11	24	2	3	0.6
ANU258 EPS	-	-	-	4	-	9	45	-	23	11	1.4	-
ANU258 LPS pk1	-	-	-	-	-	5	26	-	12	5	1.8	0.6
ANU258 LPS pk2	3	-	1	2	3	2	1	6	8	0.1	0.6	0.6
ANU258 LPS pk3	-	-	-	-	-	-	5	TR	3	0.5	0.4	0.1
ANU262 EPS	-	-	-	2	-	8	30	-	18	13	1.5	-
ANU262 LPS pk1	-	-	-	TR	-	2	10	-	3	2.7	0.9	0.8
ANU262 LPS pk2	4	-	2	3	3	3	1	9	11	0.4	0.8	1.1
ANU262 LPS pk3	TR	-	-	TR	TR	TR	10	1	8	0.4	0.4	0.2

* Data from Hanley (17)
** Data from Shatters (34)
Abreviations as given in Table III

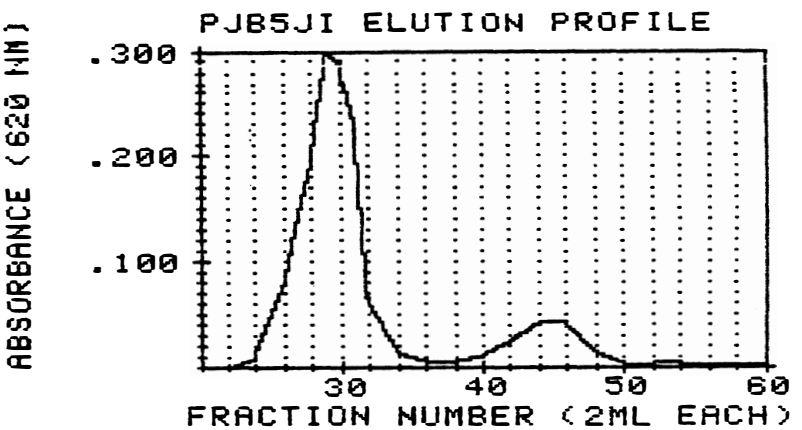

FIGURE 1
THE SEPHAROSE 4B COLUMN HEXOSE ASSAY
OF *R. leguminosarum* ANU54 pJB5JI EPS

Void volume - fraction 14 Included volume - fraction 42

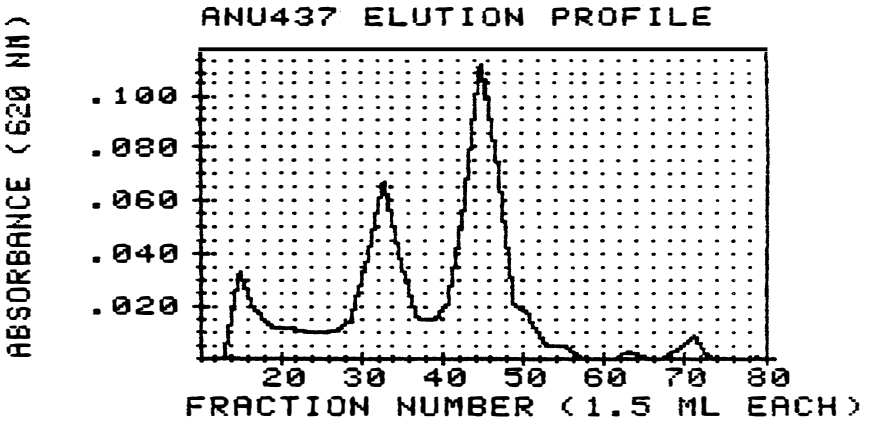

FIGURE 2
THE SEPHAROSE 4B COLUMN HEXOSE ASSAY
OF *R. trifolii* ANU437 EPS

Void volume - fraction 18 Included volume - fraction 56

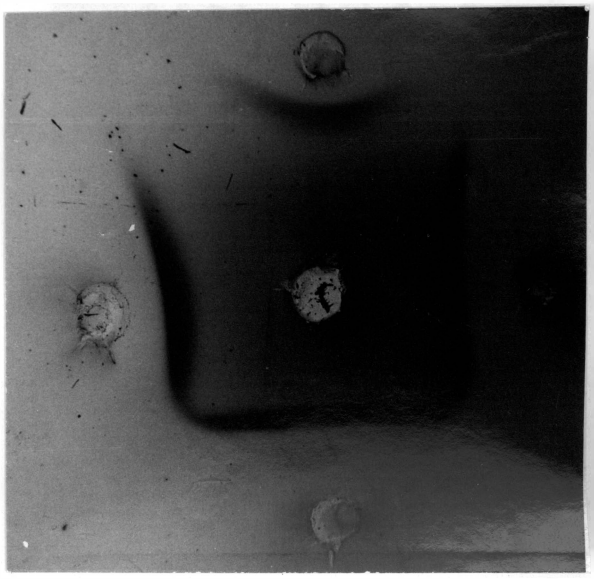

FIGURE 3
R. leguminosarum DOUBLE DIFFUSION SLIDE

Center well - ANU54 pJB5JI antiserum
Clockwise, beginning at the top well:
128C53 LPS, ANU54 LPS, ANU54 pJB5JI LPS, ANU54 pBR1AN LPS

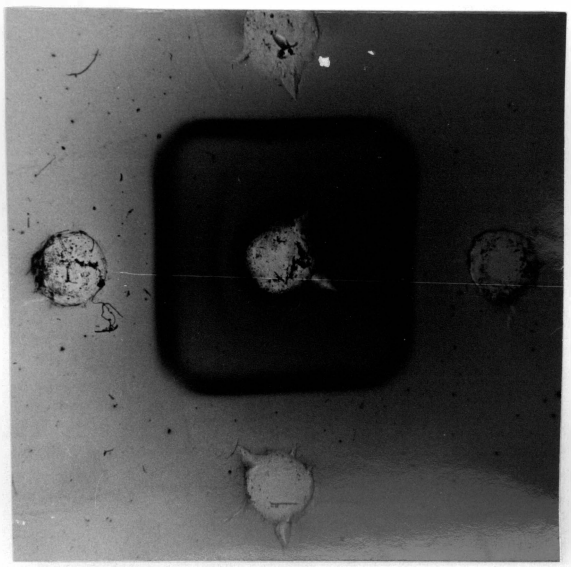

FIGURE 4

R. leguminosarum DOUBLE DIFFUSION SLIDE

Center well - ANU54 pJB5JI antiserum
Clockwise, beginning at the top well:
ANU54 pJB5JI LPS, ANU54 pJB5JI EPS pk1
ANU54 pJB5JI LPS, ANU54 pJB5JI EPS pk1

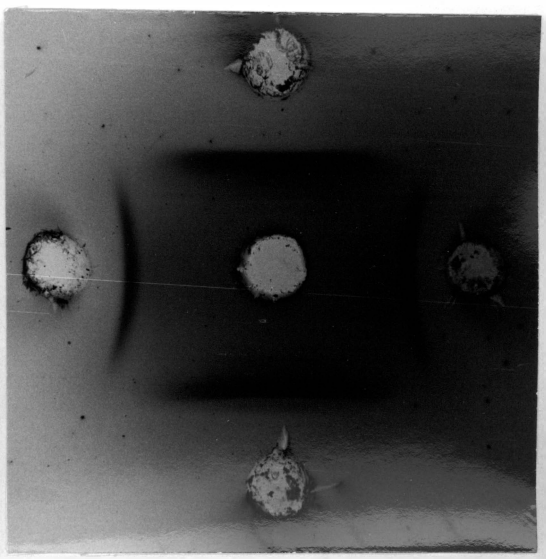

FIGURE 5

R. leguminosarum DOUBLE DIFFUSION SLIDE

Center well - ANU54 antiserum
Clockwise, beginning at the top well:
ANU54 LPS, ANU54 EPS pk1, ANU54 LPS, ANU54 EPS pk1

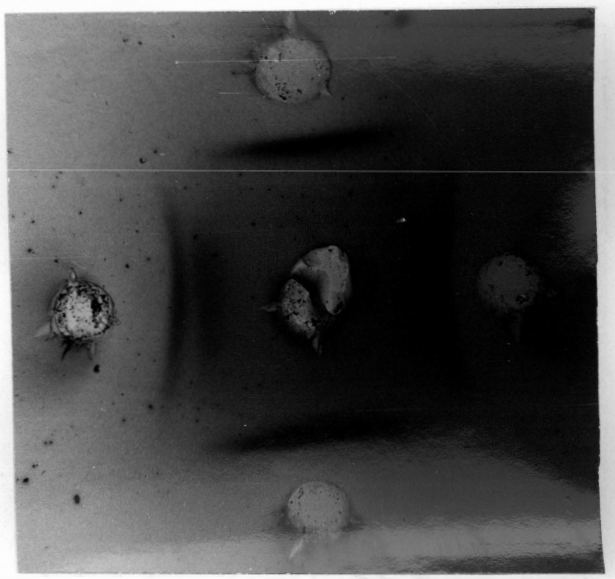

FIGURE 6

R. leguminosarum DOUBLE DIFFUSION SLIDE

Center well - ANU54 pBR1AN antiserum
Clockwise, beginning at the top well:
ANU54 pBR1AN LPS, ANU54 pBR1AN EPS pk1
ANU54 pBR1AN LPS, ANU54 pBR1AN EPS pk1

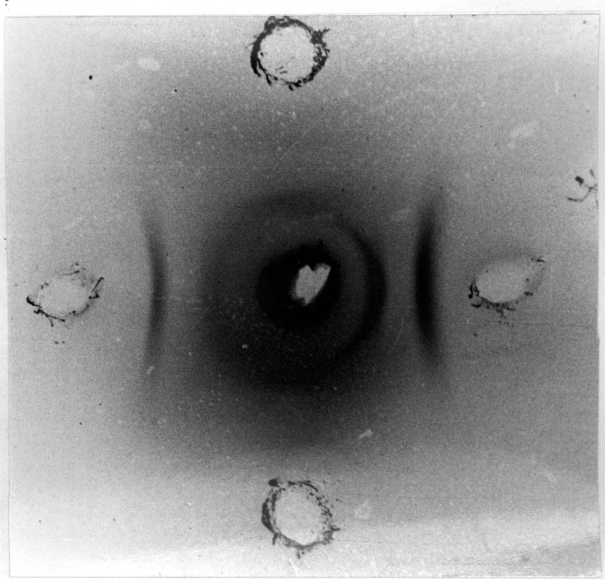

FIGURE 7

R. trifolii DOUBLE DIFFUSION SLIDE

Center well - ANU845 pRT150 antiserum
Clockwise, beginning at the top well:
ANU 845 pRT150 bacteria, ANU 843 bacteria,
ANU 845 pRT150 bacteria, ANU 843 bacteria

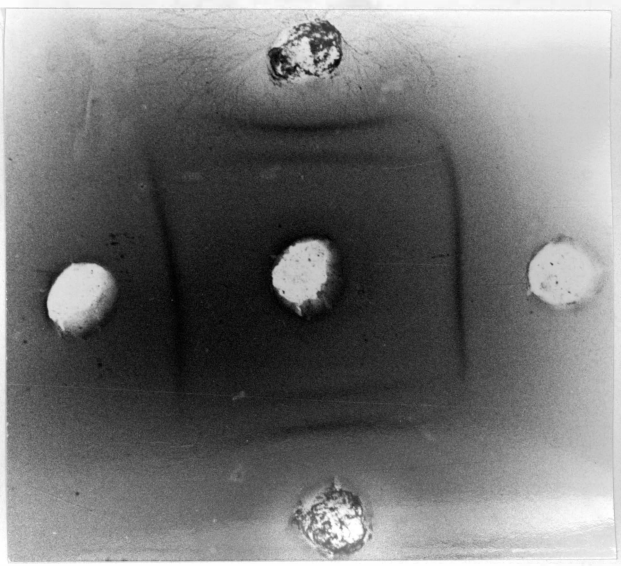

FIGURE 8

R. trifolii DOUBLE DIFFUSION SLIDE

Center well - ANU843 antiserum
Clockwise, beginning at the top well:
ANU843 bacteria, ANU845 pRT150 LPS,
ANU843 bacteria, ANU843 LPS

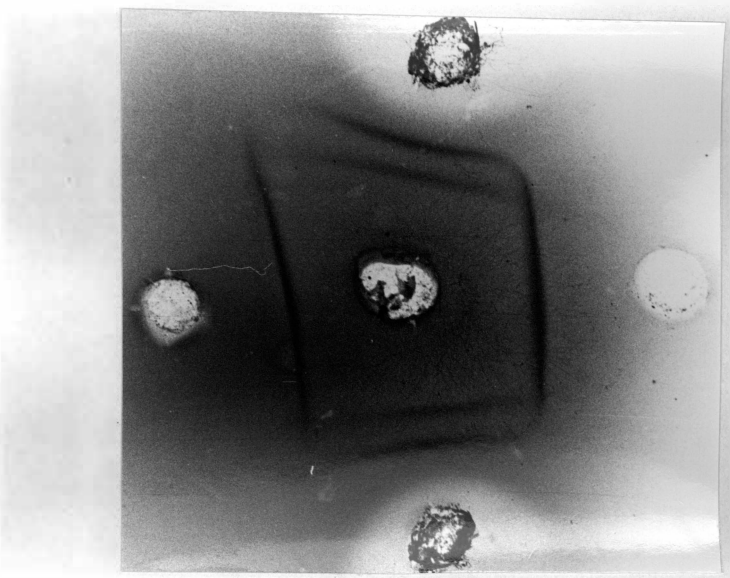

FIGURE 9

R. trifolii DOUBLE DIFFUSION SLIDE

Center well - ANU843 antiserum
Clockwise, beginning at the top well:
ANU843 bacteria, ANU845 LPS, ANU843 bacteria, ANU851 LPS

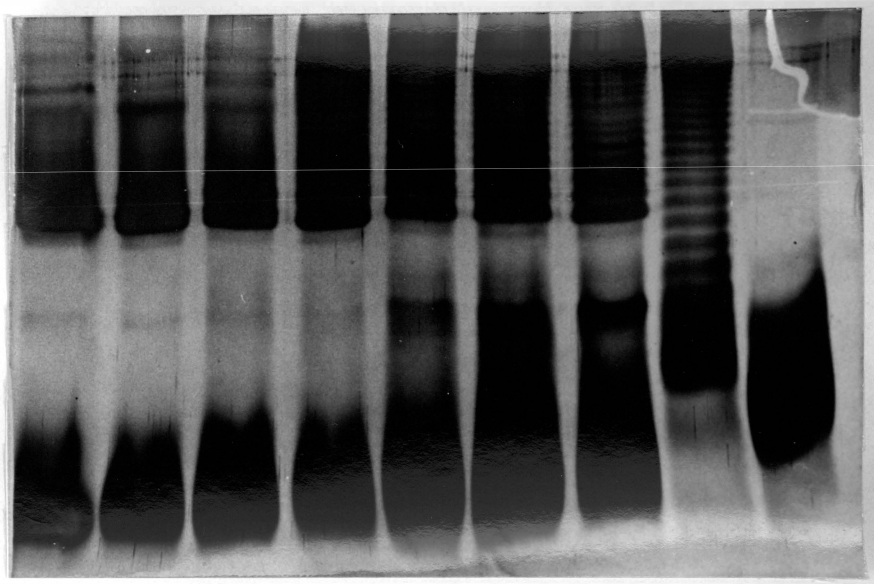

FIGURE 10

POLYACRYLAMIDE GEL ELECTROHORESIS
OF *R. leguminosarum* LPS and EPS pk1

From left to right:
Lane 1, ANU54 LPS; Lane 2, ANU54 pJB5JI LPS;
Lane 3, ANU54 pBR1AN LPS; Lane 4 128C53 LPS;
Lane 5, ANU54 EPS pk1; Lane 6, ANU54 pJB5JI EPS pk1
Lane 7, ANU54 pBR1AN EPS pk1; Lane 8 *Salmonella typhimurum*
LPS - wild type; Lane 9, *S. typhimurum* LPS - rough mutant

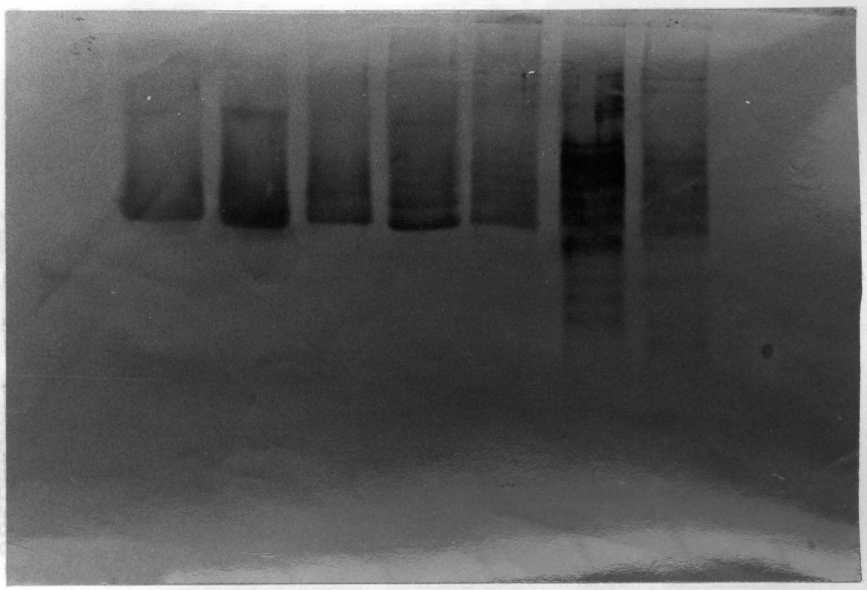

FIGURE 11

IMMUNOBLOT OF *R. leguminosarum* LPS and EPS pk1

Lanes are as described in Figure 10

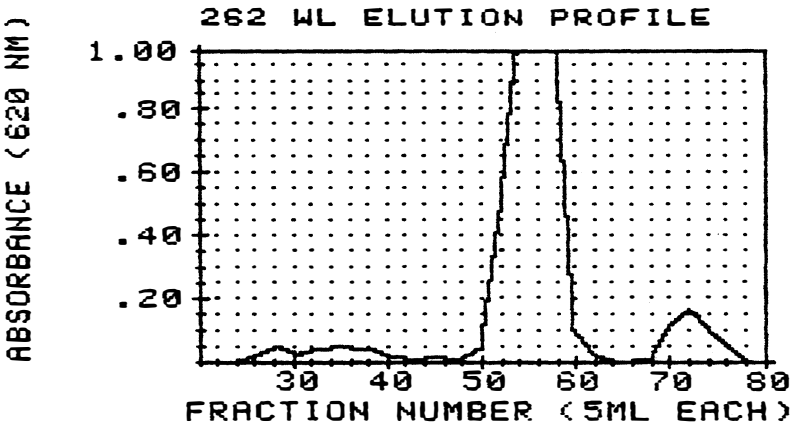

FIGURE 12

THE SEPHAROSE 4B COLUMN HEXOSE ASSAY OF
R. trifolii ANU262 Water Layer Material

Void volume - fraction 26 Included volume - fraction 78

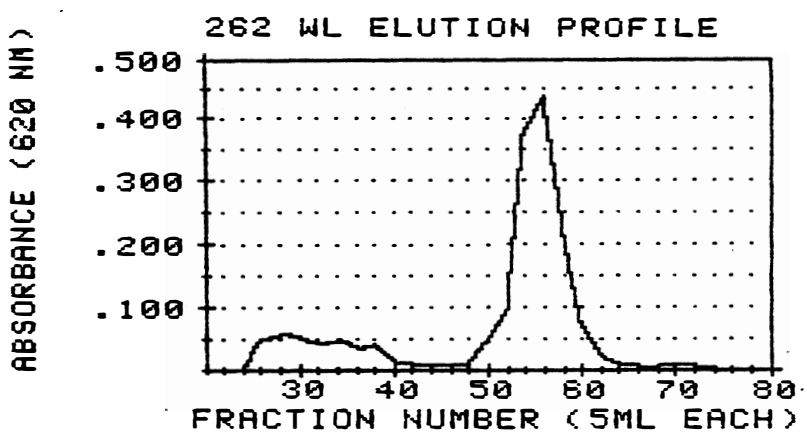

FIGURE 13

THE SEPHAROSE 4B COLUMN KDO ASSAY OF
R.trifolii ANU262 Water Layer Material

Void volume - fraction 26 Included volume - fraction 78

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